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Full Length Research Paper

Biochemical substitution of fungal xylanases for prebleaching of hardwood kraft pulp

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Xylanase enzymes of three fungi, *Aspergillus indicus, A. flavus* and *A. niveus*, were purified and characterized. The enzymes are used in the pretreatment of Hardwood kraft pulp prior to conventional alkali extraction and conventional chlorine extraction sequence (EDED process) normally used for bleaching of pulp. In the enzyme pretreated pulp when subjected to alkali extraction process the kappa number was reduced to a maximum of 5.0, 6.2 and 6.8 from 18.60 and the brightness was increased to a maximum of 43.12, 42.20 and 45.19 ISO units, respectively, from 19.83 by xylanases of *A. indicus, A. flavus* and *A. niveus*. Whereas, in the enzyme pretreated pulp, when subjected to EDED process, the maximum reduction in kappa number of 6.7, 7.2 and 7.1 and a maximum increase in brightness of 41.28, 41.06 and 41.07 ISO units, respectively, were observed in case of *A. indicus, A. flavus* and *A. niveus.*

Key words: Biobleaching, brightness, fungal xylanase, kappa number, kraft pulp, pretreatment.

INTRODUCTION

Conversion of wood into paper pulp via the Kraft process accounts for the vast majority of the paper production worldwide. Paper pulp produced by this process contains residual lignin and lignin derivatives entrapped in the pulp matrix by xylan. Bleaching is required in the kraft process to remove the residual lignin and brighten the pulp. Present day bleaching of kraft pulp uses large amounts of chlorine and chloride chemicals. The United States Environmental Protection Agency (US EPA, 1992) estimated that each year, 32 million pounds of toxic chemicals are discharged by paper mills in the Great Lakes States. These emissions are largely byproducts of chemical pulping and bleaching processes.

Biological alternatives to traditional pulp and paper industry process have been the focus of extensive research in recent years. Biopulping and biobleaching are two processes where the enzymes play an important role.

Enzymes provide a very simple and cost effective way to reduce the use of chlorine and other bleaching chemicals. It has been suggested that the hemicellulases notably endoxylanases work by hydrolyzing xylan thereby liberating entrapped lignin (Kantelinen et al., 1993; Howard et al., 2003). Hemicellulose cements cellulose to lignin and thus degradation of hemicellulose leads to mutual dissociation of the two, consequently, facilitating enhanced lignin removal.

In the present study, the xylanases obtained from *A. indicus, A. flavus* and *A. niveus* were purified, characterised and tried for pre-bleaching of *Eucalyptus grandis* hardwood kraft pulp before alkali and chlorine extraction processes.

MATERIALS AND METHODS

Microorganisms

The test fungi *A. indicus, A. flavus* and *A. niveus* were isolated from Western Ghat ecosystem by nylon net litter bag technique (Palaniswamy, 1997). The fungi were maintained on Czapek Dox agar medium (Purvis et al., 1964).

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Enzyme production

For xylanase production, the fungi were grown in Carter and Bull (Carter and Bull, 1969) medium amended with 1% (w/v) oat spelt xylan for 7 days on a rotary shaker (125 rpm) at 27° ± 2°C. After 7 days, the fungal biomass was harvested and the fungal growth was determined in terms of mycelial dry weight and protein and chitin content (Phillips and Gordon, 1989). The filtrate was centrifuged at 10,000 rpm for 20 min at 4°C and dialyzed against distilled water for overnight. The dialyzed sample was analyzed for xylanase activity, protease activity and protein content (Lowry et al., 1951).

Enzyme purification

The xylanase enzyme was purified from the culture filtrate in a stepwise process of precipitation, ion exchange chromatography and adsorption chromatography (Keskar et al., 1989). Xylanase activity and protein content were estimated after each step and a purification table was constructed.

Enzyme characterization

Properties of the xylanase enzymes viz. optimum pH, temperature, thermostability (at 37°C and 60°C), optimum substrate concentration (V_{max} and K_m) (LineWeaver and Burk, 1934) and molecular weight were determined (Laemmli, 1970) by using oat spelt xylan as substrate. The pI values of the enzymes were determined by isoelectric focussing (IEF) technique (O'Farrell, 1975).

Xylanase enzyme assay

Xylanase enzyme was assayed using oat spelt xylan (1% w/v) as substrate. The reaction mixture containing 0.5 ml of sample solution and 0.5 ml of substrate was incubated at 50°C for 30 min. The enzyme activity was determined by measuring the release of xylose using Somogyi method (Somogyi, 1952). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 µM of xylose from the substrate in 1 min under the standard condition.

Biobleaching

Unbleached pulp of *E. grandis* wood chips was obtained from Tamil Nadu Newsprint Limited (TNPL), Kagithapuram, Tamil Nadu, India. The pulp was subjected to the following treatments.

Pretreatment with xylanase enzyme: The wood pulp (2.5% consistency) was treated with various concentrations of xylanase enzyme (250, 500, 1000, 1500 and 2000 IU/ml) at 50°C for 24 h. After 24 h, the pulp was filtered and the supernatant was assayed for pH and reducing sugar content (method (Somogyi, 1952). A portion of the filtered pulp was made into hand sheets and the kappa number (TAPPI, 1990) and brightness of the hand sheets were estimated. After thorough washing in distilled water, the remaining pulp was dried and used for alkali and chlorine treatment. Pulp treated with potassium phosphate buffer (50 mM; pH 7.2) served as control.

Alkali treatment: Enzyme and buffer pretreated pulps (1% consistency) were treated with 3% sodium hydroxide at 80°C for 1 h (Christov and Prior, 1994). After the treatment, the pulp was washed with excess of water, filtered and dried. Hand sheets were prepared from the treated pulp and the kappa number and brightness of the hand sheets were determined.

Conventional chlorine extraction sequence (EDED): Enzyme and buffer pretreated pulps were subjected to conventional chlorine extraction sequence (EDED process) as recommended by Buchert et al. (1992). After the bleaching process, pulp was thoroughly washed and hand sheets were prepared. The kappa number and brightness of the hand sheets were determined.

Statistical analysis

kappa number and brightness of the pulp obtained in various treatments were analyzed by Duncan's multiple range test (Duncan, 1955).

RESULTS

In recent years, the most promising application of xylanase is in prebleaching of pulp (Ragauskas et al., 1994; Ratto et al., 1994). Xylanases are predicted to have a future market as 'bulk enzymes' in industry. To avoid release of toxic chlorinated compounds into the environment and loss of cellulose, cellulase free xylanases are used to pretreat pulp.

Enzyme production

The test fungi, *A. indicus*, *A. flavus* and *A. niveus* were grown in Carter and Bull medium amended with oat spelt xylan to test the ability of the fungi to produce xylanase enzyme. *A. indicus* produced the maximum amount of enzymes of 145.044 and 14.597 IU/ml endoxylanase and β-xylosidase, respectively. Whereas *A. flavus* produced 140.810 and 13.679 IU/ml and *A. indicus* produced only 141.091 and 5.228 IU/ml of ml endoxylanase and β-xylosidase, respectively.

Enzyme purification

The xylanase enzyme-buffer extract obtained after ethanol precipitation was purified by DEAE cellulose (batch) treatment and sephadex G-50 column chromatography (Table 1). *A. indicus* culture filtrate had 62.35 IU/mg xylanase. Ethanol precipitation increased the specific activity to 74.51 IU/mg with a purification of 1.2 folds and recovery of 39.80%. In DEAE cellulose treatment, the specific activity was increased to 154.79 IU/mg; the purification fold was 2.48 and recovery was 31.45%. In sephadex G-50 column chromatography, the seventh fraction (Figure 1a) showed maximum specific activity of 457.49 IU/mg with a purification fold of 7.30 and recovery of 11.01%. More or less similar results were

Table 1. Purification of xylanase enzyme from culture filtrates.

Fungal Species	Sample	Total volume (ml)	Activity (IU/ml)	Total activity (IU)	Protein (mg/ml)	Specific activity (IU/mg)	Purification (fold)	% Recovery
A. indicus	Culture filtrate	180.00	155.87	28057.10	2.50	62.35	1.00	100.00
	Ethanol precipitation	40.00	279.39	11175.79	3.75	74.51	1.20	39.80
	DEAE cellulose	60.00	147.05	8822.99	0.95	154.79	2.48	31.45
	Column chromatography-Sephadex G – 50 (Fraction – 7)	15.00	205.87	3088.05	0.45	457.49	7.30	11.01
A. flavus	Culture filtrate	180.00	135.29	24352.20	2.15	62.92	1.00	100.00
	Ethanol precipitation	40.00	270.57	10822.87	3.75	72.15	1.15	44.44
	DEAE cellulose	60.00	144.11	8646.53	1.00	144.11	2.29	35.51
	Column chromatography-Sephadex G – 50 (Fraction - 7)	15.00	191.17	2867.48	0.50	382.33	6.07	11.78
A. niveus	Culture filtrate	180.00	147.05	26468.96	2.00	73.52	1.00	100.00
	Ethanol precipitation	40.00	264.69	10587.59	3.00	88.23	1.20	40.00
	DEAE cellulose	60.00	161.75	9705.29	1.05	154.05	2.09	36.70
	Column chromatography-Sephadex G – 50 (Fraction - 5)	15.00	182.34	2735.10	0.60	303.90	4.12	10.33

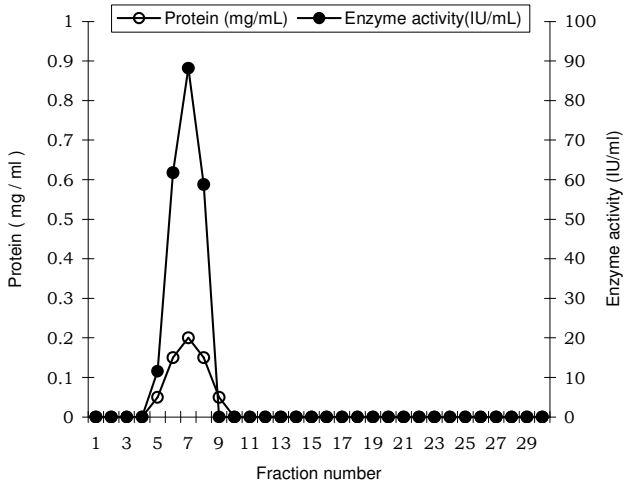

Figure 1a. Purification of xylanase enzymes from A. indicus using Sephadex G-50 column.

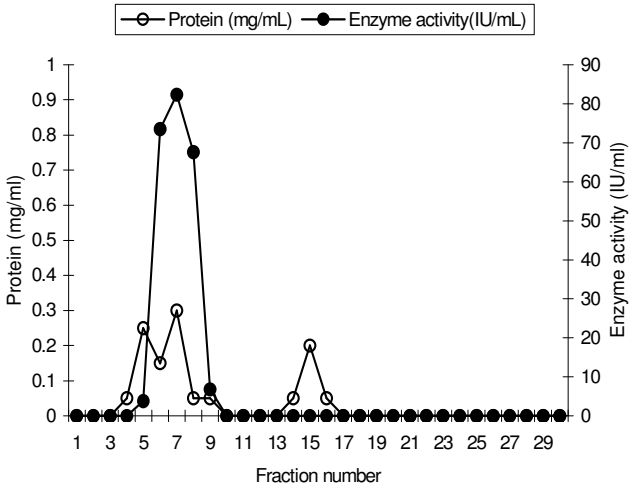

Figure 1b. Purification of xylanase enzymes from A. flavus using Sephadex G-50 column.

observed in *A. flavus* xylanase enzyme purification process. The specific activity, purification fold and per cent recovery in the culture filtrate (62.92 IU/mg, 1.00 and 100% respectively), ethanol precipitation (72.15 IU/mg, 1.15 and 44.44% respectively), DEAE cellulose treatment (144.11 IU/mg, 2.29 and 33.51%, respectively) and in sephadex G-50 column chromatography (Figure 1b: seventh fraction - 382.33 IU/mg , 6.07 and 11.78%, respectively) were more or less equal to that of *A. indicus* xylanase. The xylanase preparation of *A. niveus* showed slightly higher specific activity in culture filtrate (73.52 IU/mg), ethanol precipitation (88.23 IU/mg) and DEAE (154.05 IU/mg); but in sephadex G-50 column chromatography, the fifth fraction (Figure 1c) showed enzyme activity of 303.90 IU/mg which had purification fold of 4.12 and recovery of 10.33%.

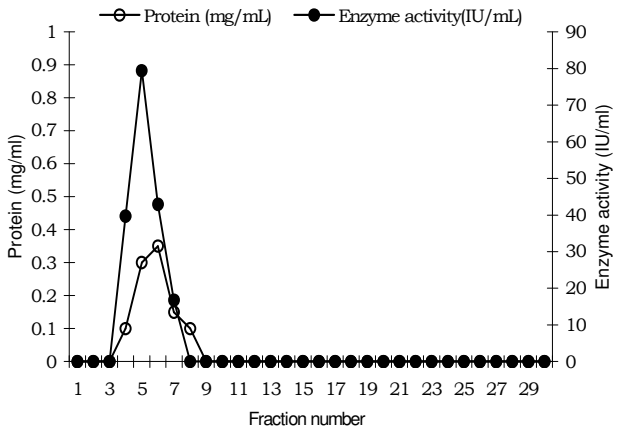

Figure 1c. Purification of xylanase enzymes from A. niveus using Sephadex G-50 column.

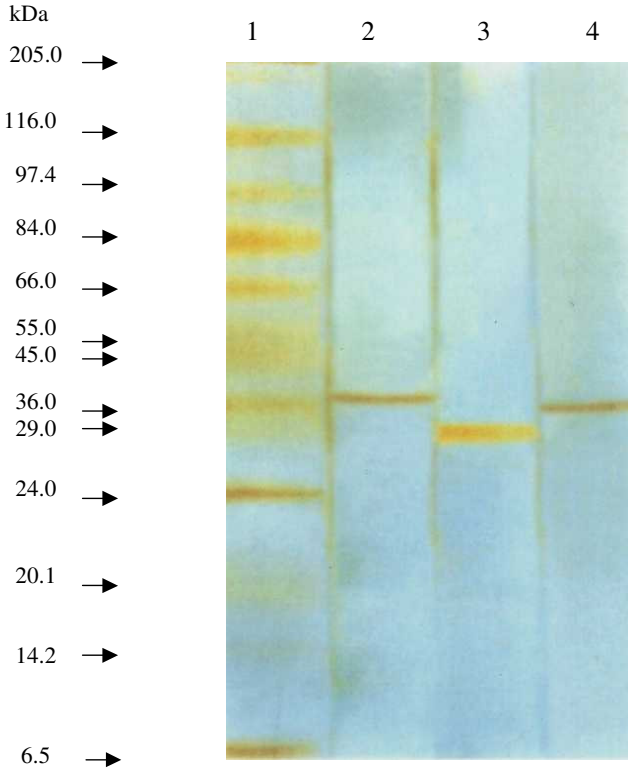

Figure 2. SDS PAGE gel showing the molecular weights of A. *indicus*, A. *flavus* and A. *niveus* xylanases. Lane 1, marker; Lane 2, A. *indicus*; Lane 3, A. *flavus*; Lane 4, A. *niveus.*

Enzyme characterization

The properties of the purified xylanase enzymes viz., optimum pH, temperature, K_m, V_{max}, molecular weight and pI were determined (Table 2). The results revealed that the xylanases obtained in the present study had optimum pH of 5 to 6 and temperature of 50°C. The enzymes were stable for three days at 37°C and at 60°C, *A. indicus*

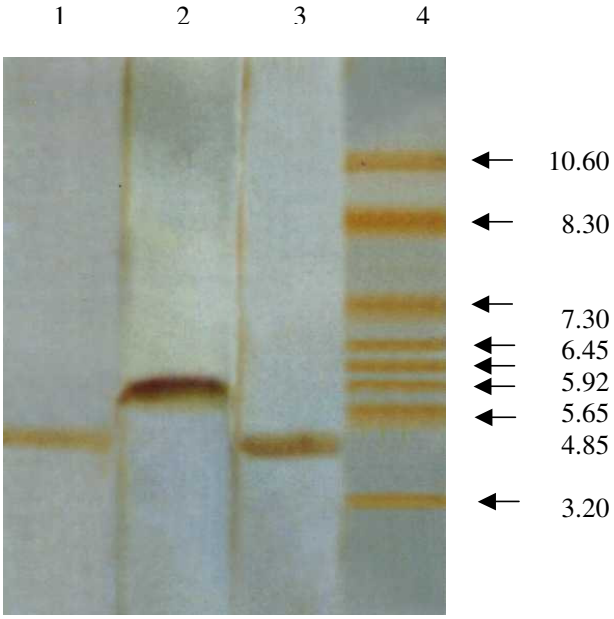

Figure 3. IEF Gel showing the pI values of A. *indicus,* A. *flavus* and A. *niveus* xylanases. Lane 1, A. *indicus;* Lane 2, A. *flavus*; Lane 3, A. *niveus*; Lane 4, marker.

enzyme was stable for 60 min and the other two for 30 min. V_{max} and K_m values were determined against xylan substrate. *A. indicus* xylanase had a higher V_{max} and K_m of 450.20 IU/mg protein and K_m was 2.50 mg/ml. *A. flavus* xylanase had a lower V_{max} of 383.45 and K_m of 0.8 mg/ml. *A. niveus* xylanase showed a still lower V_{max} values of 313.56 IU/mg protein but a higher K_m of 2.2 mg/ml respectively. The molecular weights of the xylanases were 38.7, 36.0 and 32.3 kDa for *A.indicus, A.flavus* and *A.niveus*, respectively (Figure 2), and the corresponding pI values were 4.45, 5.50 and 4.3 (Figure 3).

Biobleaching

Results of the present study implicated that pretreatment of wood pulp with fungal xylanases prior to chemical bleaching could improve the pulp quality by aiding in removal of hemicelluloses and lignin from the pulp. This process might be due to degradation of xylans, which cement the lignin together in the matrix by xylanase, so that the lignin could be released more easily (Mansfield et al., 1996) or due to decrease in the degree of polymerization of the matrix formed by xylans thus facilitating lignin diffusion out of the matrix (Paice et al., 1989).

In the present study it was observed that increasing concentrations of enzymes increased the amount of pentosans released from the pulp, which denotes the removal of hemicelluloses from the paper pulp. Maximum

Table 2. Properties of xylanase enzyme.

Properties	*A. indicus*	*A. flavus*	*A. niveus*
Optimum pH	5.0	6.0	5.0
Optimum temperature (^oC)	50	50	50
Thermostability			
37^oC (days)	3	3	3
60^oC (min)	60	30	30
V_{max} (IU / mg protein)	450.20	383.45	313.56
K_m (mg / ml)	2.50	0.80	2.20
Molecular weight (kDa) (SDS-PAGE)	38.70	36.00	32.30
PI	4.45	5.50	4.30

Table 3. Effect of xylanase enzyme from *A. indicus* pretreatment on paper quality.

Treatment / Enzyme Concentration (IU / g pulp)	Pentosans Released (%)	DMRT ranking	Kappa number	DMRT ranking	Brightness (ISO Units)	DMRT ranking
Enzyme treatment						
0	0.52	f,6	18.60	m,13	19.83	p,16
250	1.48	e,5	11.60	j,10	35.12	m,13
500	2.96	d,4	11.20	i,9	35.15	l,12
1000	3.77	c,3	10.40	h,8	36.03	k,11
1500	4.51	b,2	8.20	e,5	37.02	i,9
2000	4.58	a,1	8.20	e,5	36.21	j,10
Enzyme and alkali treatment						
0	0.37	h,8	16.50	k,11	25.59	n,14
250	0.37	h,8	8.80	f,6	41.23	e,5
500	0.37	h,8	8.80	f,6	42.87	c,3
1000	0.37	h,8	7.80	d,4	43.12	a,1
1500	0.44	g,7	5.00	a,1	43.08	b,2
2000	0.44	g,7	5.00	a,1	43.12	a,1
Enzyme and EDED treatment						
0	0.15	k,11	17.60	l,12	23.70	o,15
250	0.22	j,10	9.10	g,7	37.99	h,8
500	0.22	j,10	9.10	g,7	38.02	g,7
1000	0.30	i,9	8.20	e,5	40.98	f,6
1500	0.37	h,8	6.90	c,3	41.23	e,5
2000	0.37	h,8	6.70	b,2	41.28	d,4
CV:	0.8%		0.4%		0.0%	
p:	0.01		0.01		0.01	
SED:	0.008		0.028		0.008	
LSD (1%):	0.022		0.077		0.022	

The means followed by a common letter are not significantly different at 5% level by DMRT.

release was observed in enzyme treatment (4.14 to 4.66% at 2000 IU/g pulp concentration). Among the enzymes, xylanase from *A. niveus* yielded maximum pentosans removal. The enzyme pretreatment process itself reduced the kappa number from 18.60 to 7.40 and increased the brightness from 19.83 to 40.80 ISO units. Maximum decrease in kappa number and increase in brightness were observed in *A. niveus* xylanase treatment at 1000 to 1500 IU/g pulp enzyme concentration. When the enzyme pretreated pulp was subjected to alkali treatment, the kappa number was reduced to 5.0, 6.2 and 6.8 and the brightness was increased to 43.12, 42.20 and 45.19, respectively, by xylanases of *A. indicus, A. flavus* and *A . niveus* at 2000

Table 4. Effect of xylanase enzyme from *A. flavus* pretreatment on paper quality.

Treatment / Enzyme Concentration (IU / g ulp)	Pentosans Released (%)	DMRT ranking	Kappa number	DMRT ranking	Brightness (ISO Units)	DMRT ranking
Enzyme treatment						
0	0.52	f,6	18.60	l,13	19.83	q,17
250	1.26	e,5	14.80	i,10	29.61	n,14
500	2.66	d,4	14.00	h,9	30.12	m,13
1000	3.18	c,3	14.00	h,9	30.43	l,12
1500	4.07	b,2	8.20	c,4	40.72	e,5
2000	4.14	a,1	8.20	c,4	40.80	d,4
Enzyme and alkali treatment						
0	0.37	h,9	16.50	j,11	25.59	o,15
250	0.37	h,9	9.20	e,6	38.12	j,10
500	0.37	h,9	8.40	d,5	39.56	g,7
1000	0.37	h,9	8.20	c,4	39.98	f,6
1500	0.44	g,7	6.20	a,1	42.20	a,1
2000	0.44	g,8	6.20	a,1	42.20	a,2
Enzyme and EDED treatment						
0	0.15	j,11	17.60	k,12	23.70	p,16
250	0.15	j,11	11.30	g,8	35.42	k,11
500	0.22	i,10	10.30	f,7	38.59	i,9
1000	0.22	i,10	10.30	f,7	38.88	h,8
1500	0.22	i,10	7.10	b,2	41.01	c,3
2000	0.22	i,10	7.20	b,3	41.06	b,2
CV:	0.9%		0.9%		0.0%	
p:	0.01		0.01		0.01	
SED:	0.008		0.08		0.008	
LSD (1%):	0.022		0.22		0.022	

The means followed by a common letter are not significantly different at 5% level by DMRT

IU/g pulp enzyme concentration. When the pretreated pulp was subjected to EDED process, reduction in kappa number to 6.7, 7.2 and 7.1 and increase in brightness by 41.28, 41.06 and 41.07 ISO units respectively were observed in *A. indicus, A. flavus* and *A. niveus* xylanase pretreatments, respectively (Table 3 to 5). The DMRT analysis of the data (Table 6) revealed that pretreatment of wood pulp with xylanase enzyme of *A. indicus* at 1500 IU/g pulp concentration followed by alkali treatment was more effective in reducing the kappa number of the paper pulp, while *A. niveus* xylanase at 2000 IU/g pulp concentration followed by alkali treatment was effective in increasing the brightness of the paper pulp. For EDED process pretreatment of pulp with *A. indicus* xylanase at 2000 IU/g pulp concentration was observed to be effective.

DISCUSSION

It has been reported that xylanase purified from *A. ochraceus* NG-13 had a specific activity of 0.501 U/mg with a purification fold of 3.0 (Biswas et al., 1990). Purified xylanase from *Bacillus stearothermophilus* T-6 using sulfoxy ethyl cellulose cation exchanger had a specific activity of 213 U/mg (Fishman et al., 1995). Chandraraj and Chandra, (1996) reported that purification of xylanase from *A. fischeri* Fxnl by DEAE cellulose and DEAE sephadex column chromatography resulted in 11.5 fold purity. Egana et al. (1996) purified two types of xylanases from *Penicillium purpurogenum* using Bio-gel P-300 and CM-sephadex C-50 columns with a specific activity of 122.1 and 173.3 U/mg; the purification fold was 2.22. Purification profile of xylanases from *Cellulomonas* showed three xylanases with specific activity 923, 711 and 500 μmol/min/mg with the purification fold of 27.7, 21.3 and 15.0 (Chaudhary and Deobagkar, 1997). Compared to these observations, the xylanases purified from the culture filtrates of the test fungi in the present study were observed to have higher specific activity and purification fold next to the bacterium, C*ellulomonas.*

Earlier reports revealed that xylanase enzyme of *A. fischeri* Fxnl had optimum pH and temperature of 6.0 and 60°C respectively. $t_{1/2}$ at 50°C was 490 min and at 60°C it

Table 5. Effect of xylanase enzyme from *A. niveus* pretreatment on paper quality.

Treatment / Enzyme Concentration (IU / g pulp)	Pentosans Released (%)	\DMRTranking	Kappa number	DMRT ranking	Brightness (ISO Units)	DMRT ranking
Enzyme treatment						
0	0.52	f,6	18.60	n,14	19.83	m,18
250	1.55	e,5	10.40	k,11	38.22	j,15
500	3.03	d,4	10.20	j,10	38.56	i,14
1000	3.85	c,3	7.40	e,5	39.15	h,11
1500	4.59	b,2	7.40	e,5	40.08	f,9
2000	4.66	a,1	7.40	e,5	40.08	f,8
Enzyme and alkali treatment						
0	0.37	h,8	16.50	l,12	25.59	k,16
250	0.37	h,8	8.60	g,7	42.55	d,5
500	0.37	h,8	7.80	f,6	43.61	c,4
1000	0.44	g,7	6.90	b,2	44.12	b,3
1500	0.44	g,7	6.80	a,1	45.16	a,2
2000	0.44	g,7	6.80	a,1	45.19	a,1
Enzyme and EDED treatment						
0	0.15	k,11	17.60	m,13	23.70	l,17
250	0.15	k,11	9.90	i,9	38.67	i,13
500	0.15	k,11	9.30	h,8	39.01	h,12
1000	0.22	j,10	7.10	d,4	40.11	g,10
1500	0.30	i,9	7.00	c,3	41.03	e,6
2000	0.30	i,9	7.10	d,4	41.07	e,7
CV:	2.1%		0.4%		0.3%	
p:	0.01		0.01		0.01	
SED:	0.021		0.034		0.092	
LSD (1%):	0.057		0.093		0.25	

The means followed by a common letter are not significantly different at 5% level by DMRT.

was 8 min. It had a K_m of 4.88 mg/ml and V_{max} of 588 µmol/min/mg. The molecular weight was 31 kDa (Chandraraj and Chandra, 1996). Two types of xylanases obtained from *P. purpurogenum* were reported to have a pH optima of 5.3 and 6.0 and temperature optima of 50 and 60°C, respectively; the pI values were 7.5 and 7.8 with molecular weights of 48 and 23 kDa (Chaudhary and Deobagkar, 1997). Two types of xylanases were produced by thermotolerant *Aspergillus* FP-470 strain at 37°C and 45°C with pH optima of 6.5 and 4.3; temperature optima were 80 and 50°C; the molecular weights were 22 and 70 kDa; K_m values were 0.171 and 0.177 mg xylan/ml and V_{max} were 1.96 x 10^{-2} and 0.986 µmol/min/mg, respectively (Mendicuti Castro, 1997). The purified enzyme from *A. ochraceus* had a pH optimum of 6.0 and temperature optimum of 50°C; molecular weight was 48 kDa; K_m and V_{max} values were 1 x 10^3 M and 19.6 µmol/min/mg, respectively; $t_{1/2}$ at 45°C was 30 min (Biswas, 1990). Srinivasan and Rele (1999) have reported that fungal xylanases had optimum pH of 6.0 to 8.0, temperature of 50 to 60°C, pI of 4.4 to 6.3 and molecular mass of 24 to 35 kDa. In the present study it was observed that the *Aspergillus spp*. xylanases had pH optima of 5 to 6, temperature of 50°C, V_{max} of 313.56 to 450.20 IU/mg protein, K_m of 0.80 to 2.50 mg/ml, molecular weight of 23.3 to 35.3 kDa and pI of 4.0 to 5.6 Commercial xylanases such as Novozyme 473, VAI xylanase and Catazyme HS-10 resulted in a 3% increase in brightness (Bajpaiet al., 1994). Silva et al. (1997) reported that xylanase of *Humicola* sp. reduced the kappa number by 25% and increased the brightness by 10%. The biotreatment of bagasse pulp with xylanase from *Bacillus* sp. NCIM 59 resulted in 21% reduction in kappa number and 2.5% increase in brightness (Kulkarni and Rao, 1996). Beg et al. (2000) reported that *Streptomyces* sp. QG-11-3 xylanase pretreatment of eucalyptus kraft pulp (optimum conditions: 3.5 U/g pulp; 50°C for 2 h; pH 8.5 to 9.0) followed by chlorine treatment with 4.5% Cl_2 (optimum conditions: 50°C for 45 min; pH 2.0) reduced the kappa number of pulp from 16.08 to

Table 6. DMRT ranking for the effect of various treatments on paper quality.

Treatment Number	Treatment	Kappa number	DMRT ranking	Brightness (ISO Units)	DMRT ranking
T1	Untreated	18.60	g,9	19.80	f,10
T2	Ai 1000 A	7.80	f,8	43.12	b,4
T3	1500 A	5.00	a,1	43.08	b,5
T4	2000 A	5.00	a,1	43.12	b,4
T5	2000 E	6.70	c,3	41.28	d,7
T6	Af 1500 A	6.20	b,2	42.20	c,6
T7	2000 A	6.20	b,2	42.20	c,6
T8	1500 E	7.10	e,6	41.01	e,9
T9	2000 E	7.20	e,7	41.06	e,8
T10	An 1000 A	6.90	d,5	45.12	a,3
T11	1500 A	6.80	cd,4	45.16	a,2
T12	2000 A	6.80	cd,4	45.19	a,1
CV:		1.3%		0.1%	
p:		0.01		0.01	
SED:		0.08		0.03	
LSD (1%):		0.23		0.10	

The means followed by a common letter are not significantly different at 5% level by DMRT.
Ai, *A. indicus*; Af, *A. flavus*; An, *A. niveus*.
Xylanase enzyme; number denotes concentration of enzyme (IU/g pulp)
A, Alkali treatment; E, EDED process.

9.25 and increased the brightness from 25.50 to 50.40 ISO. A single pretreatment with the enzymes α-galactosidase, mannanase and xylanase from *Pseudomonas fluorescens* sub sp. gave the most effective kappa number reduction from 24 to 9.72 (Clarke et al., 2000).

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